

# Image Cover Sheet

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**TITLE**

INDOMETHACIN INHIBITS CIRCULATING PGE2 AND REVERSES POSTEXERCISE SUPPRESSION OF  
NATURAL KILLER CELL ACTIVITY

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# Indomethacin inhibits circulating PGE<sub>2</sub> and reverses postexercise suppression of natural killer cell activity

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**Rhind, Shawn G., Greg A. Gannon, Masatoshi Suzui, Roy J. Shephard, and Pang N. Shek.** Indomethacin treatment inhibits circulating PGE<sub>2</sub> and reverses postexercise suppression of natural killer cell activity. *Am. J. Physiol.* 276 (Regulatory Integrative Comp. Physiol. 45): R1496–R1505, 1999.—Natural killer (NK) cells are important in combating viral infections and cancer. NK cytolytic activity (NKCA) is often depressed during recovery from strenuous exercise. Lymphocyte subset redistribution and/or inhibition of NK cells via soluble mediators, such as prostaglandin (PG) E<sub>2</sub> and cortisol, are suggested as mechanisms. Ten untrained (peak O<sub>2</sub> consumption = 44.0 ± 3.5 ml · kg<sup>-1</sup> · min<sup>-1</sup>) men completed at 2-wk intervals a resting control session and three randomized double-blind exercise trials after the oral administration of a placebo, the PG inhibitor indomethacin (75 mg/day for 5 days), or naltrexone (reported elsewhere). Circulating CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> NK cell counts, PGE<sub>2</sub>, cortisol, and NKCA were measured before, at 0.5-h intervals during, and at 2 and 24 h after a 2-h bout of cycle ergometer exercise (65% peak O<sub>2</sub> consumption). During placebo and indomethacin conditions, exercise induced significant ( $P < 0.0001$ ) elevations of NKCA (>100%) and circulating NK cell counts (>350%) compared with corresponding control values. With placebo treatment, total NKCA was suppressed (28%;  $P < 0.05$ ) 2 h after exercise, and a postexercise elevation (36%;  $P = 0.02$ ) of circulating PGE<sub>2</sub> was negatively correlated ( $r = 0.475$ ,  $P = 0.03$ ) with K-562 tumor cell lysis. NK counts were unchanged in the postexercise period, but at this stage CD14<sup>+</sup> monocyte numbers were elevated ( $P < 0.0001$ ). Indomethacin treatment eliminated the postexercise increase in PGE<sub>2</sub> concentration and completely reversed the suppression of total and per CD16<sup>+</sup>56<sup>+</sup> NKCA 2 h after exercise. These data support the hypothesis that the postexercise reduction in NKCA reflects changes in circulating PGE<sub>2</sub> rather than a differential lymphocyte redistribution.

cellular immunity; cortisol; cyclooxygenase inhibition; cytokines; cytotoxicity; eicosanoids; lymphocyte subsets

THE BODY DEPLOYS NATURAL killer (NK) cells as its first line of defense in combating viral infections and the development of specific cancers (3, 6). Strenuous or prolonged exercise may reduce immune surveillance; suggested mechanisms include lymphocyte subset redistribution and/or the release of various soluble mediators (39). Despite considerable research (4, 35, 41), the precise mechanisms and physiological significance of

exercise-induced changes in NK cytolytic activity (NKCA) remain unclear (19, 41).

Controversy centers on whether the postexercise suppression of NKCA reflects changes in the cytolytic capacity of individual NK cells or whether changes in NKCA simply mirror alterations in the circulating NK cell count (5, 36, 40). Several investigators have observed a correspondence between fluctuations in NKCA and NK cell concentrations (19, 46, 50), supporting the hypothesis that the exercise-induced modulation of cytotoxicity reflects no more than a redistribution of effector lymphocyte subsets within the peripheral blood. However, postexercise depression of NKCA is not always accompanied by a reduction in the number of circulating NK cells (35, 40, 53), suggesting possible downregulation by endocrine and/or paracrine mediators.

Possible negative modulators of NKCA include prostaglandin (PG) E<sub>2</sub> and cortisol (13, 20), both of which can be elevated after exercise (4, 10). The inhibitory action of PGE<sub>2</sub> on NK cells is mediated via elevation of intracellular cAMP (9, 23) after stimulation of cell surface PGE<sub>2</sub> receptors that are positively coupled to adenylyl cyclase (22). Increased cAMP is thought to suppress NKCA by interfering with specific steps along the lytic pathway (7, 30, 45). This sequence of events can be countered by the nonsteroidal anti-inflammatory drug indomethacin, which blocks PGE<sub>2</sub> biosynthesis via inhibition of cyclooxygenase activity (57). Maximal suppression of PG production occurs with doses between 50 and 150 mg (1). In addition to the independent effects of PGE<sub>2</sub> on NKCA, low circulating levels of PGE<sub>2</sub> can synergize with endogenous glucocorticoids to inhibit cell-mediated immune function (16, 34).

The magnitude and kinetics of changes in plasma PGE<sub>2</sub> concentration with exercise are poorly characterized (26), and evidence concerning the putative role of endogenous PGE<sub>2</sub> in exercise-induced suppression of NKCA is conflicting (36, 40). Interpretation of previous investigations is complicated by a lack of data on circulating PGE<sub>2</sub>, failure to utilize randomized, double-blind, placebo-controlled protocols, and failure to include a nonexercise control session for comparison with matched exercise responses. In this context we aimed to extend earlier investigations, using a double-blind, counterbalanced and placebo-controlled design, relating NK cell counts and NKCA throughout the exercise and recovery period to plasma concentrations of PGE<sub>2</sub> and cortisol. Our primary hypotheses were that a

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strenuous 2-h bout of cycle ergometer exercise would induce increased circulating PGE<sub>2</sub> concentrations and postexercise suppression of NKCA and that these responses would be reversed by 5 days of oral indomethacin treatment.

## METHODS

**Subjects.** Ten untrained, but recreationally active [peak O<sub>2</sub> consumption ( $\dot{V}O_{2\text{peak}}$ ) =  $44.0 \pm 3.5$  (SD) ml·kg<sup>-1</sup>·min<sup>-1</sup>], nonsmoking men (mean  $\pm$  SD:  $26.3 \pm 5.4$  yr of age,  $79.3 \pm 10.3$  kg body mass,  $1.78 \pm 0.07$  m height) volunteered to participate in the study under conditions approved by the University of Toronto and Defence and Civil Institute of Environmental Medicine Human Experimentation Committees. At preliminary medical examination, subjects were excluded if they had a history of gastrointestinal ulcers or other known forms of sensitivity to nonsteroidal anti-inflammatory drugs. Other specific criteria for exclusion included a history of allergies and acute or chronic infection.

**Experimental design.** The study consisted of five laboratory visits: 1) clinical, physical, and anthropometric assessment, 2) a nonexercise, resting control condition, and 3) three double-blind exercise tests ordered according to a randomized block design (placebo, indomethacin, and naltrexone). For the purposes of this study, only the resting control, placebo, and indomethacin trials are considered. A separate component of the study involving administration of the opioid antagonist naltrexone is described elsewhere (18).

**Physical assessment.** After medical approval, but  $\geq 1$  wk before the control condition,  $\dot{V}O_{2\text{peak}}$  and heart rate were determined on a mechanically braked cycle ergometer (Ergo-med 818E, Monark, Stockholm, Sweden). Subjects performed a progressive test at a pedal cadence of 70 rpm (an initial loading of 60 W, with 25 W/min increments). Volitional exhaustion was reached in 8–12 min. Expired gas, collected breath-by-breath, was analyzed for respiratory minute volume and O<sub>2</sub> consumption with use of a metabolic measurement cart (model 2900C, SensorMedics, Yorba Linda, CA). A heart rate monitor (Vantage XL, Polar, Port Washington, NY) was used to record heart rates at 5-s intervals. The work rate needed to elicit 65%  $\dot{V}O_{2\text{peak}}$  was determined for each subject from a plot of work rate vs. O<sub>2</sub> consumption.

**Control and experimental trials.** Within 2 wk of the physical assessment, subjects underwent resting control observations followed by three randomized, counterbalanced exercise trials at intervals of  $\geq 2$  wk. This design allowed for a systemic clearance of drug metabolites and ensured that a subject's hematologic status was not compromised from previous blood sampling. On each test day, subjects reported to the laboratory at 0700–0730, having fasted overnight and abstained from strenuous physical activity for 36 h. They were immediately instrumented with a heart rate monitor and a 21-gauge intravenous catheter (Insyte Vascular Access, Becton-Dickinson, Sandy, UT). To standardize metabolic conditions, each subject consumed 1.1 MJ (250 kcal) of a clinical nutritional supplement (Ensure Plus, Abbott Laboratories, Saint-Laurent, PQ, Canada) immediately after collection of the initial blood sample.

The control session served to familiarize subjects with personnel, protocol, equipment, and the laboratory environment used for exercise testing. Beginning at 0730–0800, after ~30 min of seated rest, serial blood samples were collected according to the same schedule as in exercise trials.

The two exercise trials involved administration of placebo or indomethacin before a 2-h bout of cycle ergometer exercise at a pedal cadence of 60 rpm and an intensity adjusted to

elicit 65% of the individual's  $\dot{V}O_{2\text{peak}}$ . O<sub>2</sub> consumption and heart rate were monitored at 15-min intervals during exercise, and the load was adjusted as necessary to maintain the required intensity of effort. Participants were encouraged to consume 1.0–1.5 liters of water during trials to minimize hemoconcentration. Sterile glass Vacutainers (Becton-Dickinson, Franklin Lakes, NJ) containing the necessary preservatives and anticoagulants were used to collect 45-ml blood samples at 0 (baseline), 0.5, 1, 1.5, 2, 4, and 24 h relative to the start of exercise.

**Drug administration and pharmacokinetics.** Beginning 5 days before the two exercise trials, subjects were given, in a double-blind fashion, four capsules of identical appearance to be taken orally each morning with breakfast; each capsule contained an inert placebo (180 mg of lactose; Novopharm, Scarborough, ON, Canada) or indomethacin (75 mg of Indocid SR, Merk Frosst, Mississauga, ON, Canada). Compliance was controlled by observation of drug ingestion on scheduled test days. Approximately 90% of orally administered Indocid is absorbed via the gastrointestinal tract within 2–4 h, and the selected dose would have yielded peak plasma concentrations of 1–2 µg/ml (1).

**Hematologic analyses.** Determinations of total leukocyte counts, three-cell differential counts (granulocytes, monocytes, and lymphocytes), Hb, and hematocrit were performed on K<sub>3</sub>EDTA-treated blood with use of an automated hematology analyzer (model JT, Coulter Electronics, Hialeah, FL). The formulas of Dill and Costill (11) were used to correct all blood cell counts and hormone concentrations to resting blood and plasma volumes, respectively.

**Immunophenotyping by flow cytometry.** NK cells (CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup>) and monocytes (CD45<sup>+</sup>CD14<sup>+</sup>) were enumerated by dual-parameter immunophenotyping with use of combinations of monoclonal antibodies conjugated to FITC or phycoerythrin (PE). Briefly, 100-µl samples of EDTA-whole blood were incubated with saturating amounts of fluorochrome-conjugated monoclonal antibodies, as previously described (44). Stained cell suspensions were enumerated on a FACScan flow cytometer equipped with a 488-nm air-cooled argon-ion laser by using standard operating methods (Becton-Dickinson, San Jose, CA). Daily instrument calibration was performed with a mixture of monosized FITC- and PE-conjugated and unconjugated latex particles (4.8-µm CalibRITE beads) and AutoCOMP software (Becton-Dickinson). Isotype-negative controls (anti-IgG<sub>1</sub>-FITC/IgG<sub>1</sub>-PE) and anti-CD4-FITC/CD8-PE double-stained whole blood samples served to optimize forward- and side-scatter gains. Electronic compensation was adjusted to eliminate spectral overlap between fluorescence (FL) 1, FL2, and FL3 channels. Sample data were acquired on the day of collection and subsequently analyzed using CellQUEST software (Becton-Dickinson).

**Isolation of peripheral blood mononuclear cells.** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples (143 USP U/10 ml blood) by density gradient centrifugation for 30 min (20°C, 400 g) over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The mononuclear cell band was carefully aspirated, washed, and reconstituted to a concentration of  $2.0 \times 10^7$  cells/ml in 10% FCS-RPMI 1640.

**K-562 tumor cell culture.** The NK-sensitive K-562 tumor cell line (American Type Culture Collection, Rockville, MA) served as target cells for the cytolytic assays. The cell line was maintained in a continuous suspension of RPMI 1640 culture medium containing 10% FCS, 1% (wt/vol) penicillin-streptomycin, 20 mM HEPES (pH 7.3), and 2 mM L-glutamine (GIBCO Life Technologies, Burlington, ON, Canada) in 25-cm<sup>2</sup> tissue culture flasks at 37°C in a humidified 5% CO<sub>2</sub>

incubator (Revco Ultima, VWR Scientific, Toronto, ON, Canada). To ensure that cells were in the logarithmic phase of growth, cultures were split into  $3\text{--}5 \times 10^9$  cells/ml on a 24-h schedule 3 days before the experiment. Cell viability as assessed by trypan blue dye exclusion was typically  $>90\%$ .

**Tumor cell labeling.** K-562 tumor cells were first washed twice in 10 ml of RPMI 1640 medium without FCS and centrifuged for 5 min ( $20^\circ\text{C}$ , 400 g). The cells were then resuspended at a concentration of  $1 \times 10^7$ /ml and labeled using a stable lipophilic membrane dye (PKH-26, Sigma Chemical, St. Louis, MO). A volume of 0.5 ml of the target cell suspension was added rapidly to 0.5 ml of PKH-26 ( $4 \mu\text{M}$ ) in a  $12 \times 75\text{-mm}$  polystyrene culture tube. After incubation at  $25^\circ\text{C}$  for 2–5 min, the reaction was stopped by the addition of 1 ml of 100% FCS for 1 min. After centrifugation ( $20^\circ\text{C}$ , 400 g) for 5 min, cells were washed three times in 10 ml of supplemented RPMI 1640 medium and resuspended to a final concentration of  $2 \times 10^5$  cells/ml.

**NK cytolytic assay.** Spontaneous NKCA was assessed by a nonradiometric in vitro flow cytometric assay (42). Plasma membrane integrity of the PKH-26-labeled K-562 tumor target cells was determined using the DNA-intercalating dye propidium iodide (PI; Sigma Chemical). Briefly, 100  $\mu\text{l}$  of freshly isolated PBMC (effectors,  $1 \times 10^7$  cells/ml) were gently mixed with 100  $\mu\text{l}$  of PKH-26-labeled K-562 tumor cells (targets,  $2 \times 10^5$  cells/ml) and 25  $\mu\text{l}$  (1  $\mu\text{g}/\text{ml}$ ) of PI solution at an effector-to-target ratio of 50:1. Cell mixtures were centrifuged for 5 min ( $20^\circ\text{C}$ , 50 g) to promote optimal effector-to-target cell conjugation and then incubated for 4 h at  $37^\circ\text{C}$  in a humid 5%  $\text{CO}_2$  atmosphere. The assay was stopped by addition of cold cell wash to the cultures. Samples were placed on ice until same-day acquisition.

PKH-26<sup>+</sup> target cells were defined flow cytometrically and gated via a histogram of FL2 fluorescence. A minimum of 5,000, live-gated PKH-26<sup>+</sup> target cell events (corresponding to  $\geq 200,000$  list mode events) were collected per sample. Dead K-562 cells were differentiated from live K-562 cells on the basis of the FL3 fluorescence of PI. Spontaneous target cell death was determined by incubating PKH-26-labeled cells with 25  $\mu\text{l}$  of PI but with no effector cells. Percent specific lysis was calculated by subtracting the mean percentage of spontaneously dead target cells from the percentage of target cells killed in the test sample (mean of triplicate values). The corresponding absolute number of dead target cells was calculated by multiplying the percent lysis by the total number of target cells used in a given assay. The intra-assay coefficient of variation (CV) was consistently  $\leq 4\%$  among triplicate samples, and the between-trial CVs for the same subject were typically  $\leq 5\%$ .

**Biochemical analyses.** Venous blood samples for cortisol determination were drawn into prechilled, 3-ml heparinized glass Vacutainers (Becton-Dickinson, Oakville, ON, Canada) and placed on ice. Samples were immediately centrifuged for 15 min ( $4^\circ\text{C}$ , 1,000 g), harvested, and frozen at  $-80^\circ\text{C}$  for future analysis. Plasma cortisol concentrations were determined using a commercial  $^{125}\text{I}$ -cortisol solid-phase RIA kit (ICN Biomedicals, Costa Mesa, CA). The intra- and interassay CVs were  $<10\%$ .

Blood samples for determination of PGE<sub>2</sub> were collected into ice-cold 5-ml (siliconized) Vacutainers containing 10 mg/ml EDTA and indomethacin (Sigma Chemical) at 5  $\mu\text{g}/\text{ml}$  blood. Solid-phase sorbent extraction of PGE<sub>2</sub> from indomethacin-treated plasma samples was performed using Amprep C<sub>18</sub> octadecylsilyl silica reverse-phase affinity chromatography minicolumns (Amersham) (24). Purified samples were eluted with ethyl acetate. The organic phase was evaporated to

dryness under  $\text{N}_2$  gas, and the residue was reconstituted in 150  $\mu\text{l}$  of assay buffer. PGE<sub>2</sub> concentration was determined by competitive enzyme immunoassay (BIOTRAK, Amersham Life Science, Buckinghamshire, UK) with a linear range of 0–32 pg/well. The PGE<sub>2</sub> antiserum had a sensitivity of 0.8 pg/well (equivalent to 16 pg/ml) and the following cross-reactivities: PGA<sub>2</sub>, 0.2%; PGB<sub>2</sub>, 1.0%; PGD<sub>2</sub>, 1.0%; PGE<sub>1</sub>, 7.0%; PGF<sub>2 $\alpha$</sub> , 4.3%; 6-keto-PGF<sub>1 $\alpha$</sub> , 5.4%; and 13,14-dihydro-15-keto-PGE<sub>2</sub>,  $<0.1\%$ . Aliquots of 50  $\mu\text{l}$  of PGE<sub>2</sub>-specific antibody standards and unknown samples were added to 96-well microtiter plates and incubated for 3 h at  $2\text{--}8^\circ\text{C}$ . Optical density was read at a wavelength of 450 nm by using an automated microplate photometer (model EL340, BIO-TEK Instruments, Winooski, VT). The intra- and interassay CVs were 9.3 and 14.6%, respectively.

**Statistical analyses.** Values are means  $\pm$  SE unless otherwise noted. To determine circadian effects, resting control data were analyzed separately for each phase of the experiment by using univariate ANOVA repeated across sampling times. Possible effects of trial order were excluded by a two-way (order  $\times$  time) ANOVA. Statistical significance of changes in leukocyte and lymphocyte subsets, NKCA, cortisol, and PGE<sub>2</sub> concentrations was analyzed using a 3 (control, placebo, indomethacin)  $\times$  7 (sampling times) factorial design. When the *F* ratio showed significant interaction effects, specific post hoc pairwise multiple contrast comparisons were computed to identify sources of differences between time points. Nominal degrees of freedom are reported along with *F* values. An  $\alpha$  level of 0.05 was accepted as indicating significance. The Geisser-Greenhouse adjustment of epsilon for degrees of freedom was used to minimize type I error. Linear regressions were calculated by the method of least squares. Percent change was determined intraindividually as follows:  $100\% \times (\text{posttest value} - \text{baseline value})/\text{baseline value}$ . All statistical calculations were performed using StatView and SuperANOVA microcomputer software packages (SAS Institute, Cary, NC).

## RESULTS

**Physiological response to acute exercise.** No significant differences in metabolic measures were detected over time ( $P = 0.85$ ) or between placebo and indomethacin exercise conditions ( $P = 0.65$ ). Subjects worked at power outputs of  $127 \pm 14$  and  $125 \pm 12$  W during placebo and indomethacin trials, respectively, eliciting an average of 65% of their individual  $\text{VO}_{2\text{peak}}$  during both trials. A significant main effect of condition [ $F(2,18) = 85.13$ ,  $P < 0.001$ ] was apparent for heart rate; steady-state exercise heart rates during the placebo trial ( $157.9 \pm 8.8$  beats/min) were on average 11 beats/min higher than those recorded during the indomethacin trial ( $146.4 \pm 9.33$  beats/min).

**Leukocyte subset counts.** Initial resting values for total peripheral blood leukocytes and leukocyte subset counts did not differ significantly between trials (Table 1). The concentrations of circulating leukocytes, monocytes, and granulocytes showed no significant fluctuations over time during resting control observations. A significant main effect of time [ $F(6,54) = 4.03$ ,  $P < 0.02$ ] was observed for lymphocyte concentration during the control condition; pairwise mean contrasts traced the source of variation to a significantly ( $P < 0.01$ ) elevated lymphocyte count after 4 h (at 1200) of seated rest. Significant condition  $\times$  time interaction

Table 1. Comparison of leukocyte subset counts during control and exercise conditions

Subset	Time, h							P (ANOVA effect, condition × time)
	Rest	Exercise					Recovery	
	0	0.5	1.0	1.5	2.0	4.0	24	
Leukocytes								
Control	5.56 ± 1.77	5.85 ± 1.62	5.77 ± 1.72	5.84 ± 1.61	5.94 ± 1.54	6.08 ± 1.19	6.40 ± 2.32	0.0001
Placebo	5.54 ± 0.88	7.92 ± 1.55*	8.04 ± 1.28*	9.57 ± 2.25†	13.2 ± 3.82†	15.0 ± 4.47†	5.95 ± 1.48	
Indomethacin	5.37 ± 1.34	7.19 ± 2.30*	7.59 ± 2.39*	9.17 ± 2.56†	12.3 ± 3.22†	13.4 ± 3.48†	6.26 ± 1.71	
Lymphocytes								
Control	2.10 ± 0.42 (37.0)	2.13 ± 0.41 (37.0)	2.04 ± 0.44 (34.7)	2.31 ± 0.42 (37.1)	2.22 ± 0.42 (36.3)	2.39 ± 0.38‡ (38.5)	2.32 ± 0.38 (37.2)	0.0001
Placebo	2.08 ± 0.39 (37.8)	3.18 ± 0.74* (39.8)	3.16 ± 0.52* (38.3)	3.46 ± 0.48† (35.5)	3.97 ± 0.71† (30.2)	1.98 ± 0.69* (14.1)	2.24 ± 0.33 (39.1)	
Indomethacin	2.00 ± 0.37 (37.0)	2.88 ± 0.50* (40.5)	3.02 ± 0.58* (39.7)	3.48 ± 0.86† (36.6)	3.80 ± 0.91† (30.0)	1.87 ± 0.27 (14.7)	2.08 ± 0.42 (34.6)	
Monocytes								
Control	0.38 ± 0.06 (7.01)	0.35 ± 0.05 (7.21)	0.40 ± 0.05 (7.75)	0.39 ± 0.04 (7.20)	0.38 ± 0.10 (7.10)	0.36 ± 0.05 (7.30)	0.34 ± 0.09 (7.22)	0.0001
Placebo	0.39 ± 0.07 (7.09)	0.62 ± 0.16† (7.80)	0.62 ± 0.15† (7.70)	0.70 ± 0.19† (7.35)	0.88 ± 0.22† (6.81)	0.76 ± 0.31† (5.11)	0.41 ± 0.08 (7.08)	
Indomethacin	0.38 ± 0.06 (7.23)	0.52 ± 0.13† (7.64)	0.53 ± 0.15† (7.26)	0.61 ± 0.16† (6.98)	0.70 ± 0.08† (6.46)	0.60 ± 0.19† (4.80)	0.39 ± 0.08 (6.37)	
Granulocytes								
Control	3.20 ± 1.47 (53.4)	3.46 ± 1.35 (59.3)	3.43 ± 1.42 (59.6)	3.45 ± 1.53 (58.7)	3.35 ± 1.29 (58.3)	3.34 ± 1.05 (56.2)	3.85 ± 2.07 (58.5)	0.0001
Placebo	3.20 ± 0.78 (57.8)	4.27 ± 1.13 (54.4)	4.46 ± 1.02 (56.4)	5.63 ± 2.05* (59.6)	8.61 ± 3.45† (64.9)	12.7 ± 4.52† (83.4)	3.46 ± 1.39 (57.3)	
Indomethacin	3.15 ± 1.23 (57.7)	3.95 ± 1.83 (54.8)	4.25 ± 2.05 (56.3)	5.32 ± 2.12* (59.4)	7.95 ± 2.44† (65.9)	11.3 ± 3.41† (83.4)	4.02 ± 1.60 (59.6)	

Values are means ± SD expressed as  $\times 10^9/l$ ; data in parentheses are percentages of total leukocytes ( $n = 10$ ). Significant differences between exercise conditions vs. control: (\* $P < 0.05$ ; † $P < 0.0001$ ). ‡Significant differences within control condition vs. time 0:  $P < 0.05$ .

effects were observed for absolute numbers of each of the leukocyte subsets listed in Table 1 (all variables  $P < 0.0001$ ). Exercise induced a sustained mobilization of circulating leukocyte subsets during placebo and indomethacin conditions, displaying marked lymphocytosis (100%), granulocytosis (300%), and monocytosis (165%) compared with resting control values. The exercise-induced granulocytosis was significantly ( $P < 0.05$ ) less pronounced during the indomethacin condition than during the placebo condition at 2 and 4 h. During the indomethacin condition, circulating lymphocyte counts fell to ~80% ( $P < 0.05$ ) of the corresponding control values 2 h after exercise (Table 1). A highly significant interaction effect [ $F(12,108) = 18.2$ ,  $P < 0.0001$ ] emerged for CD14<sup>+</sup> monocyte counts. Pairwise contrast tests between control and placebo conditions revealed significantly elevated monocyte concentrations at all time points during (all  $P < 0.001$ ) and after exercise ( $P < 0.05$ ). Indomethacin treatment blunted the exercise-induced monocytosis ( $P < 0.05$ ) at all time points during exercise. The 33% postexercise increase in CD14<sup>+</sup> monocyte counts under placebo conditions was completely abolished under the influence of indomethacin (see Fig. 2A).

**NK cell counts and cytolytic activity.** Circulating CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> NK cell counts showed a significant main effect of time [ $F(6,54) = 3.16$ ,  $P < 0.05$ ] during the resting control condition. This was traced to a >20% increase in NK counts over baseline at 4 and 24 h. Cycle ergometer exercise induced significant intertrial differences in the circulating number [ $F(12,108) = 27.8$ ,  $P <$

0.0001] and percentage [ $F(12,108) = 30.6$ ,  $P < 0.001$ ] of CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> NK cells (Fig. 1A). After 2 h of exercise, there was a 340% increase ( $P < 0.0001$ ) in NK cell counts during the indomethacin trial and a 390% ( $P < 0.0001$ ) increase in the placebo trial compared with control values. At 2 h after exercise, NK cell counts were reduced 25% during the placebo trial compared with control, yet in the indomethacin condition values did not differ significantly from control (Fig. 1A).

NKCA data are summarized in Fig. 1, B and C. Figure 1B shows the total NKCA (percent lysis) per fixed number of PBMC unadjusted for changes in the proportion of circulating NK cells and monocytes. Control NKCA showed no significant main effect of time, despite a rising trend over the 4-h sample period. Total peripheral blood NKCA showed a significant interaction effect [ $F(12,108) = 18.9$ ,  $P < 0.0001$ ], with peak increases of 100% ( $P < 0.0001$ ) during the indomethacin trial and 66% ( $P < 0.0001$ ) during the placebo trial, after 1.5 h of exercise. At 2 h after exercise, NKCA was significantly ( $P < 0.05$ ) reduced by 28% during the placebo trial but was unchanged in the indomethacin trial, in contrast to the corresponding control values. Pairwise contrasts revealed significantly higher NKCA, at all exercise time points, during the indomethacin trial than during the placebo trial (all  $P < 0.05$ ). Moreover, in vivo indomethacin treatment eliminated the significant postexercise suppression of NKCA seen during the placebo trial.

To adjust for variations in the circulating proportion of NK cells and monocytes during exercise, NKCA was

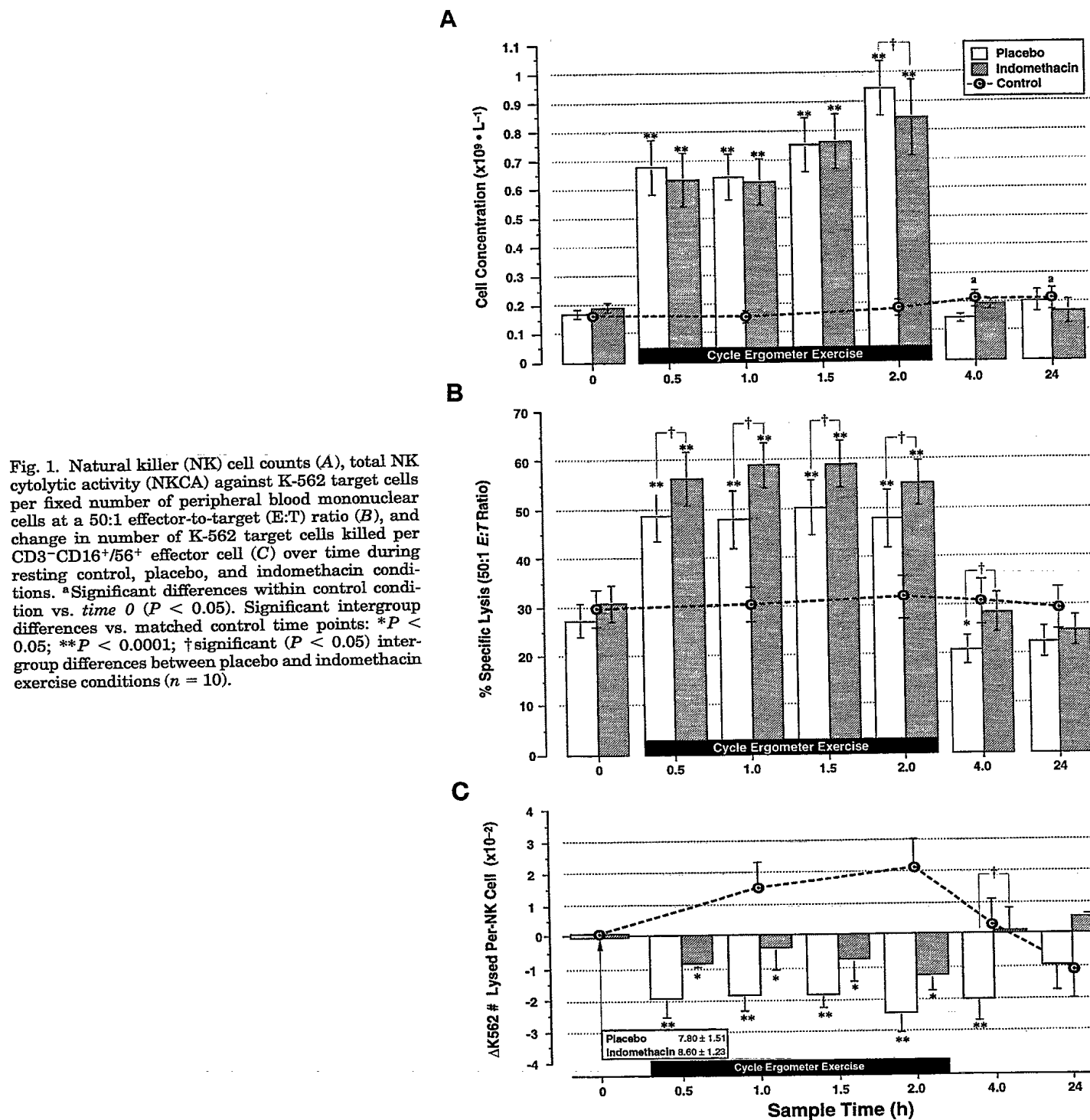


Fig. 1. Natural killer (NK) cell counts (A), total NK cytolytic activity (NKCA) against K-562 target cells per fixed number of peripheral blood mononuclear cells at a 50:1 effector-to-target (E:T) ratio (B), and change in number of K-562 target cells killed per CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> effector cell (C) over time during resting control, placebo, and indomethacin conditions. \*Significant differences within control condition vs. time 0 ( $P < 0.05$ ). Significant intergroup differences vs. matched control time points: \* $P < 0.05$ ; \*\* $P < 0.0001$ ; †significant ( $P < 0.05$ ) intergroup differences between placebo and indomethacin exercise conditions ( $n = 10$ ).

calculated as the number of lysed K-562 target cells per CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> cell with use of the following formula: per NKCA = number of dead K-562 target cells / [(%CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> PBMC) × (total PBMC count - CD14<sup>+</sup> PBMC count)]. When NKCA was adjusted on this basis, no statistically significant main effect of time [ $F(6,54) = 1.52$ ,  $P = 0.25$ ] was apparent during the resting control trial, but there was a significant interac-

tion effect [ $F(12,108) = 3.15$ ,  $P < 0.05$ ] between conditions. However, the exercise-induced increase in NKCA was no longer apparent. In fact, during the placebo trial the number of killed target cells was significantly (all  $P < 0.0001$ ) decreased (up to 50%) relative to control at all time points during exercise, and it remained significantly ( $P < 0.0001$ ) suppressed 2 h after exercise (Fig. 1C). During the indomethacin trial, per NKCA was also

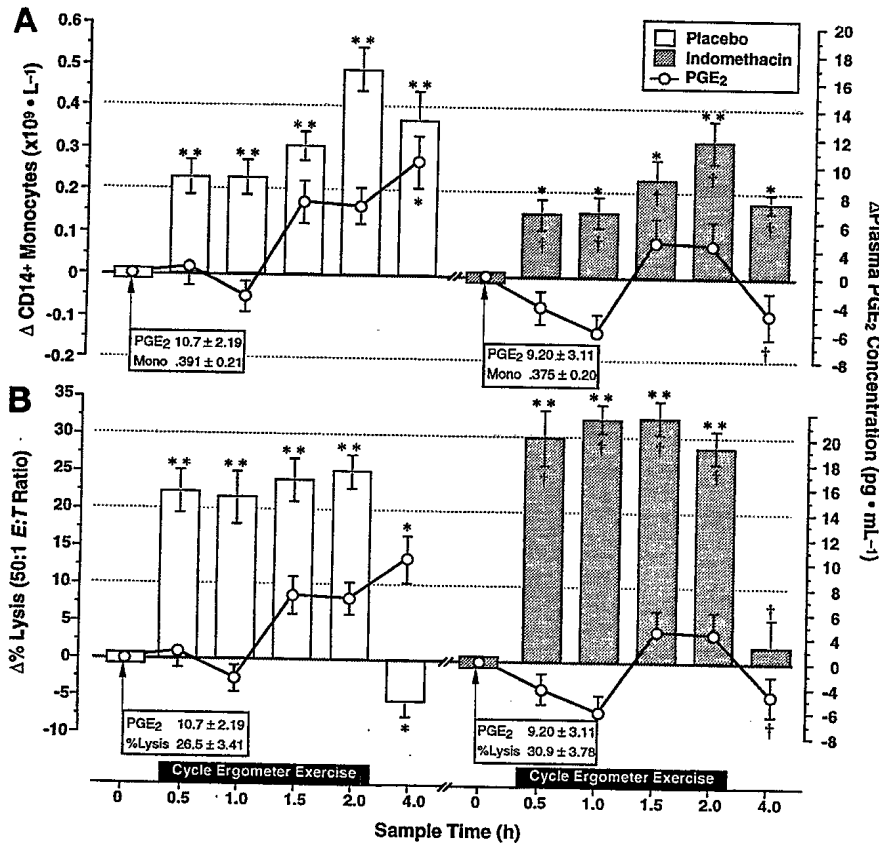


Fig. 2. Exercise-induced changes in concentration of circulating prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) vs. change in CD14<sup>+</sup> monocyte counts (A) and change in total NKCA (B) during placebo and indomethacin conditions. Values (means  $\pm$  SE) are expressed as change relative to resting (time 0) values, as shown in insets. Significant changes in PGE<sub>2</sub> concentration, monocyte counts, and NKCA within exercise conditions vs. time 0: \* $P < 0.05$ ; \*\* $P < 0.0001$ ; †significant ( $P < 0.05$ ) intergroup differences between placebo and indomethacin exercise conditions ( $n = 10$ ). Data for 24-h time points were omitted, since no significant differences in measured variables were detected.

significantly (all  $P < 0.001$ ) suppressed during exercise, but the 2- and 24-h recovery values did not differ from control values. Despite an apparent trend toward higher per NKCA with exercise during the indomethacin trial, pairwise contrasts revealed that the only significant difference in per NKCA between placebo and indomethacin conditions was at 2 h after exercise ( $P < 0.05$ ; Fig. 1C).

**Plasma PGE<sub>2</sub> concentrations.** Figure 2 displays the kinetic changes in PGE<sub>2</sub> concentration relative to changes in CD14<sup>+</sup> monocyte counts and total NKCA. Resting plasma PGE<sub>2</sub> concentrations were within the expected normal range (3–15 pg/ml) (24) and did not differ significantly between exercise conditions, averaging  $10.7 \pm 2.19$  and  $9.20 \pm 3.11$  pg/ml for the placebo and indomethacin conditions, respectively. Changes in PGE<sub>2</sub> concentrations showed a significant main effect of time [ $F(6,54) = 4.49$ ,  $P < 0.01$ ]. A significant interaction effect [ $F(12,108) = 2.15$ ,  $P < 0.05$ ] was traced to elevated (36%;  $P < 0.02$ ) PGE<sub>2</sub> relative to control values in the placebo trial 2 h after exercise (Fig. 2A). No significant differences in PGE<sub>2</sub> were noted at any other time points. Postexercise differences in PGE<sub>2</sub> between conditions were eliminated during the indomethacin trial. Plasma PGE<sub>2</sub> concentrations were negatively correlated ( $r = 0.48$ ,  $P = 0.03$ ) with the number of dead K-562 tumor cells (as a dependent variable) 2 h after exercise (Fig. 3A). The kinetics of PGE<sub>2</sub> concentration paralleled changes in CD14<sup>+</sup> monocyte counts and were found to be positively related ( $r = 0.56$ ,  $P = 0.01$ ; Fig. 3B).

**Plasma cortisol concentrations.** Initial baseline (at 0800) levels of total cortisol (11.5–14.2  $\mu$ g/dl; Fig. 4) were within the expected normal range of 5–20  $\mu$ g/dl and did not differ significantly between sessions. A

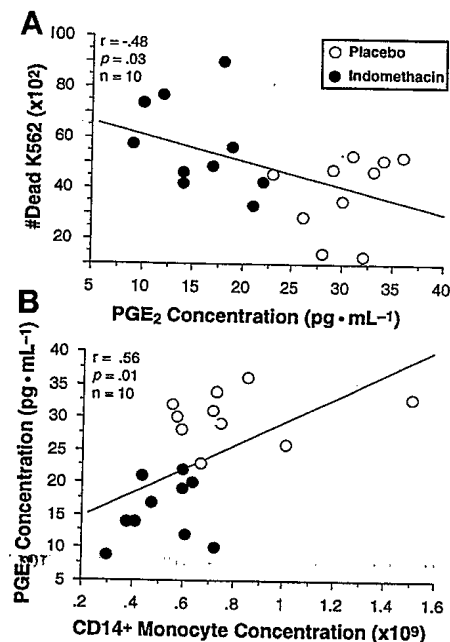


Fig. 3. Scatterplots of relationship between circulating PGE<sub>2</sub> concentration and number of dead K-562 tumor target cells per assay tube (A) and CD14<sup>+</sup> monocyte counts (B), as measured 2 h after exercise by linear regression.



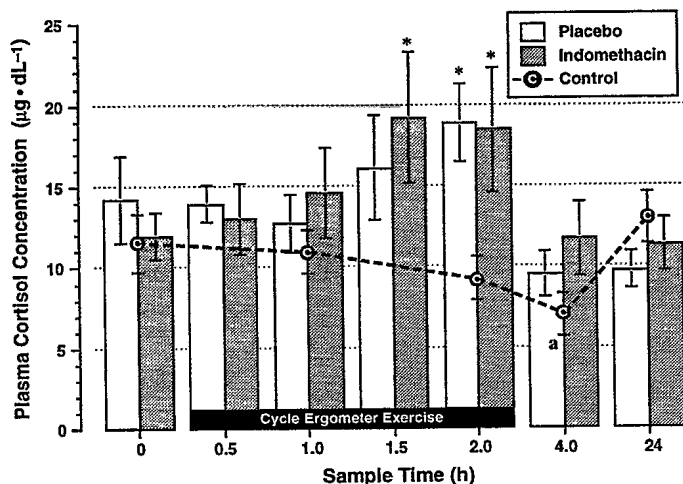


Fig. 4. Plasma cortisol concentrations during control, placebo, and indomethacin conditions. \*Significant differences within control condition vs. time 0 ( $P < 0.05$ ); \*significant ( $P < 0.05$ ) within-condition differences vs. control ( $n = 10$ ).

significant main effect of time was seen during the control condition [ $F(6,42) = 4.69$ ,  $P < 0.01$ ], with plasma cortisol values decreasing (37%) by 4 h of observation (from  $11.5 \pm 1.8$  µg/dl at 0800 to  $7.0 \pm 1.3$  µg/dl at 1200). A significant interaction effect [ $F(12,84) = 1.85$ ,  $P = 0.05$ ] was also found. Relative to corresponding control values, pairwise mean contrasts showed significant ( $P < 0.01$ ) elevations (peak value =  $18.6$  µg/dl) of cortisol at 1.5 and 2 h of exercise during the indomethacin trials and at 2 h during the placebo trial. No significant differences in cortisol concentration were observed between placebo and indomethacin trials, and under both conditions, cortisol levels had returned to normal by 2 h after exercise.

## DISCUSSION

The results of this randomized, double-blind, placebo-controlled study indicate that a 5-day oral treatment with the prostaglandin inhibitor indomethacin augments the total unadjusted NKCA of the peripheral blood compartment during prolonged exercise and also reverses the suppression of NKCA 2 h after exercise. These results confirm prior exercise trials (32, 40, 53) providing new evidence that postexercise suppression of total peripheral blood NKCA and per CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> NKCA are associated with elevated plasma concentrations of PGE<sub>2</sub> and that the suppression is reversed if PGE<sub>2</sub> levels are reduced by administration of indomethacin. The postexercise immunosuppression does not appear to result from NK cell redistribution or the solitary inhibitory action of cortisol, since removal of PGE<sub>2</sub> by indomethacin treatment restores NKCA without altering NK cell counts or circulating cortisol levels.

**Modulation of circulating PGE<sub>2</sub> and total NKCA.** This investigation is the first to directly relate plasma PGE<sub>2</sub> concentrations to circulating NK cell numbers and cytolytic activity during prolonged exercise. Prior evidence regarding changes in PGE<sub>2</sub> levels during exercise is conflicting: increases in circulating PGE<sub>2</sub>

have been seen after a marathon run (10), but not after an 8-h triathlon (26). Meanwhile, others have demonstrated that moderate cycle ergometer exercise (75%  $\dot{V}O_{2peak}$ ) stimulates intramuscular PGE<sub>2</sub> release (37), yet plasma PGE<sub>2</sub> concentrations remain unchanged after progressive cycle ergometer exercise to exhaustion (31). Such discordant findings may reflect the inherent difficulties of isolating endogenous prostanooids and/or a lower sensitivity of earlier assay methodologies (24).

The current findings of a 70–80% increase in unadjusted NKCA throughout exercise, with a 28% drop below baseline 2 h after exercise (placebo condition) are typical of the cytolytic response to prolonged cycle ergometer exercise (19). Our results confirm the findings of Pedersen et al. (40), who demonstrated that 1 h of cycle ergometer exercise (75%  $\dot{V}O_{2peak}$ ) induced a 60–70% increase in NKCA per fixed number of PBMC (50:1 effector-to-target cell ratio) followed by a 30–35% decrease 2 h after exercise. Also in accord with the current findings, this group showed that oral indomethacin treatment (50 mg, 3 times/day for 2 days) potentiates exercise-induced NKCA (>100% above rest) and abolishes the postexercise suppression of NKCA without significantly modifying NK cell numbers (40). Our results strengthen the suggestion that PGE<sub>2</sub> is necessary and sufficient for mediating postexercise inhibition of NKCA by demonstrating an inverse relationship between circulating concentrations of this mediator and the number of lysed tumor cells. In addition, the positive association observed between increases in circulating CD14<sup>+</sup> monocyte counts and circulating levels of PGE<sub>2</sub> strongly supports previous indications (40, 53) that monocyte-derived PGE<sub>2</sub> is an important physiological downregulator of NKCA after exercise.

In contrast to the current findings and those of Pedersen et al. (40), in vivo (150 mg/day for 2 days) (5) and in vitro (1 µg/ml) (36) indomethacin treatment did not significantly attenuate the postexercise suppression of total NKCA in experienced runners after 1.0 or 2.5 h of running. Furthermore, when NKCA was expressed on a per-cell basis, the postexercise depression of NKCA was no longer apparent, even in indomethacin-free cultures (36). There seem to be several possible explanations for these disparate results. Differences in exercise mode (cycling vs. running) and duration (1 or 2 vs. 2.5 h) are an obvious source of variance between studies.

Disagreement between studies may also relate to the choice of sample population. Well-trained subjects often display greater resting NKCA than untrained individuals (19, 41), although the concentration of circulating NK cells may be similar in trained and untrained groups (12, 35). The mechanism of chronic training-induced enhancement of NKCA is unknown. It could reflect the removal of, or a decrease in sensitivity to, inhibitory factors such as PGE<sub>2</sub> (44, 53). Consistent with this notion, exercise-induced increases in plasma PGE<sub>2</sub> and the sensitivity of lymphocytes to the inhibitory action of PGE<sub>2</sub> are significantly lower in chroni-

cally exercised rats than in sedentary animals (32, 58). Similarly, naive human subjects show a greater sensitivity to PGE<sub>2</sub> than do conditioned volunteers in response to various forms of physical stress (21). Furthermore, recent evidence suggests that significant postexercise suppression of NKCA may occur only when exercise exceeds the ventilatory threshold (49). Thus it can be postulated that well-conditioned runners may produce less PGE<sub>2</sub> or are possibly more refractory to the inhibitory actions of endogenous PGs than untrained individuals, such as those in the present investigation. Additional studies are necessary to confirm or refute this explanation, since published reports (5, 36) involving indomethacin treatment in conditioned runners did not assay PGE<sub>2</sub> concentrations. Alternatively, regular exercise may alter NKCA by modifying cytokine expression (19, 43).

Another potential source of variation between studies is the age of participants. The mean ages of subjects in the current study and the study of Pedersen et al. (40) were  $\leq 30$  yr, whereas the mean age of the marathon runners described by Nieman et al. (36) was  $38.7 \pm 1.5$  yr, and the matched control group was even older ( $45.3 \pm 2.3$  yr). Aging may be an important discriminator, since evidence suggests that it is associated with 1) increases in total NKCA and per NKCA (38), 2) decreases in plasma PGE<sub>2</sub> levels (8), and 3) a reduced sensitivity of circulating lymphocytes to PGE<sub>2</sub>-mediated inhibition (22).

*Change in NKCA per cell.* When expressed on a CD3-CD16<sup>+</sup>/56<sup>+</sup> per-cell basis, postexercise suppression of NKCA was also abolished by treatment with indomethacin. Surprisingly, per-cell activity was depressed throughout the entire exercise period under placebo and indomethacin conditions. Despite a trend toward higher per-cell activity with indomethacin treatment than with placebo, no statistically significant differences between conditions were observed during exercise. Because PGE<sub>2</sub> levels did not rise significantly until after exercise, the acute reduction in cytolytic capacity during exercise would appear to be mediated by PG-independent mechanisms. Such a finding reinforces the view that the regulation of NKCA is multifactorial and that a variety of mediators are likely involved in exercise-induced modulation of NK cell function (18, 39).

NKCA is a result of a fine balance between positive and negative signals induced through distinct sets of receptors activating or inhibiting cytolytic activity (6). The inhibitory action of cAMP on NKCA is well documented (30, 59). Elevation of intracellular cAMP suppresses NKCA by impairing NK-target cell recognition (54) and by reducing effector-to-target cell conjugation (33, 45), providing an important shut-off mechanism for the NK lytic pathway. Such feedback inhibition is required for the cell to continue to function. NK cells need to replenish their stores of cytolytic molecules and to reset the receptor and enzyme systems of the cytolytic response so they can respond anew to subsequent tumor cell contacts (59). In addition to PGE<sub>2</sub>, many agents that increase cAMP are potent regulators of

cytokine release (55). For example,  $\beta_2$ -adrenoceptor stimulation by epinephrine elevates cAMP (27) and downregulates the synthesis of several NK cell-stimulatory cytokines, including interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , and interleukin-12, while also provoking the release of the immunosuppressive cytokine interleukin-10 (14, 15). Furthermore, acute exercise rapidly upregulates  $\beta$ -adrenoceptor density on circulating NK cells (2). Therefore, it can be hypothesized that exercise-elicited sympathetic activation, with the systemic release of catecholamines, contributes to the acute suppression of NKCA via enhanced cAMP production and subsequent changes in cytokine production.

The immunomodulatory properties of indomethacin are not restricted to their inhibition of the cyclooxygenase system and PG production (57). Indomethacin also interferes with a number of other physiological processes, including alteration of second-messenger pathways (55) and cytokine production (52). For example, basal or exercise-induced catecholamine secretion can be directly modified by indomethacin treatment (25), and this compound has been shown to decrease  $\beta$ -adrenoceptor density and sensitivity to catecholamine stimulation (17). Such an effect is supported by the decreased heart rate response observed with exercise during the indomethacin trial. Therefore, an indomethacin-induced decrease of  $\beta$ -adrenergic activation may have led to reduced cAMP production, resulting in the dampening of cAMP-mediated immunosuppression. In addition, indomethacin is known to upregulate production of several NK cell-stimulatory cytokines (52). Taken together, these results suggest that indomethacin-mediated immunopotentiality may provide an explanation for the trend toward higher NKCA during exercise with indomethacin treatment. Future investigations are needed to more fully elucidate the complex interactions between cAMP-enhancing agents, cytokine release, and immunoregulation by exercise.

*Circulating cortisol response and NKCA.* Exercise-induced secretion of cortisol may have contributed to the overall postexercise lymphocytopenia during placebo and indomethacin conditions. An important action of glucocorticoids is their ability to interfere with cellular adhesion and migration (2). Because we did not detect any significant differences in the number of circulating NK cells relative to the control condition during the postexercise period, cortisol does not appear to have had a major impact on NK cell mobilization. This conclusion is supported by recent demonstrations that circulating cortisol levels are unrelated to the magnitude or the duration of postexercise changes in NK cell counts, although they can reduce postexercise monocytosis (47, 48).

In addition to their potential effects on lymphocyte redistribution, long-term exposure to pharmacological doses of natural and synthetic glucocorticoids can inhibit NKCA directly (20, 28, 29). Similar to PGE<sub>2</sub>-induced increases in cAMP, large doses of glucocorticoids can interfere with normal NK-target cell recognition and the triggering of lytic machinery (56), including reduced synthesis of granzyme A (60). By contrast,

physiological doses of glucocorticoids may stimulate, rather than inhibit, NKCA in rats (29). Although there have been suggestions that plasma cortisol levels are increased by indomethacin treatment (51), the present results do not support such an effect. Nevertheless, we cannot exclude the possibility that PGE<sub>2</sub> and cortisol act synergistically to downregulate NKCA during exercise, as in other forms of stress (16, 34). However, in the absence of PGE<sub>2</sub>, exercise-induced increases in cortisol are insufficient to suppress NKCA, since indomethacin treatment restored NKCA of exercising subjects to resting levels.

**Conclusion.** Our data are consistent with the hypothesis that in vivo treatment with the PG inhibitor indomethacin fully reverses the postexercise increase in PGE<sub>2</sub> and associated suppression of NKCA. Because neither the percentage nor the concentration of circulating CD3<sup>+</sup>CD16<sup>+</sup>/56<sup>+</sup> NK cells differed significantly between placebo and indomethacin treatments during the postexercise period, the attenuation of NK suppression observed after oral indomethacin treatment appears to be specific to single-cell activity and is not the result of differential lymphocyte redistribution. This conclusion is supported by earlier animal (12, 32) and human studies (40, 53). Increases in circulating cortisol may contribute to these inhibitory effects but appear insufficient to suppress NKCA in the absence of PGE<sub>2</sub>. The contribution of other immunoinhibitory mediators, including cytokines, to exercised-induced suppression of NKCA remains to be evaluated.

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